

Diversity of RuBisCO gene responsible for CO₂ fixation in an Antarctic moss pillar

Ryosuke Nakai · Takashi Abe · Tomoya Baba · Satoshi Imura · Hiroshi Kagoshima · Hiroshi Kanda · Yuji Kohara · Akiko Koi · Hironori Niki · Katsuhiko Yanagihara · Takeshi Naganuma

Received: 23 December 2011 / Revised: 16 May 2012 / Accepted: 16 May 2012 / Published online: 7 June 2012
© The Author(s) 2012. This article is published with open access at Springerlink.com

Abstract Antarctic “moss pillars” are lake-bottom biocenoses that are primarily comprised of aquatic mosses. The pillars consist of distinct redox-affected sections: oxidative exteriors and reductive interiors. Batteries of SSU rRNA genotypes of eukaryotes, eubacteria, and cyanobacteria, but no archaea, have been identified in these pillars. However, rRNA-based phylogenetic analysis

provides limited information on metabolic capabilities. To investigate the microorganisms that have the potential for CO₂ fixation in the pillars, we studied the genetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39)—an enzyme involved in CO₂ fixation. PCR clone libraries targeting all forms of the RuBisCO large subunit-encoding gene were constructed and 1,092 clones were randomly sequenced. Phylogenetic analysis indicated that proteobacterial form IA operational RuBisCO units (ORUs) were detected at the same frequency as the cyanobacterial form IB ORUs. Surprisingly, the form IA ORU, which was closely related to the sequences from deep-sea environments, was detected from all moss pillar sections. The form IB ORU related to Bryophyta, considered to be derived from moss, was identical to the sequence of *Leptobryum* sp. isolated from Lake Hotoke-Ike where the pillars were found. Moreover, certain cyanobacterial ORUs were found exclusively in the exterior of the pillar, whereas form II ORUs related to chemolithoautotrophic sulfur oxidizers and purple sulfur bacteria were found exclusively in the interior. No forms IC, ID, or III RuBisCO genes were detected. This is the first report demonstrating that bacteria with the potential for CO₂ fixation and chemoautotrophy are present in the Antarctic moss pillar ecosystem.

Electronic supplementary material The online version of this article (doi:10.1007/s00300-012-1204-5) contains supplementary material, which is available to authorized users.

R. Nakai · A. Koi · T. Naganuma (✉)
Graduate School of Biosphere Science, Hiroshima University,
1-4-4 Kagamiyama, Higashi, Hiroshima 739-8528, Japan
e-mail: takn@hiroshima-u.ac.jp

R. Nakai
Research Fellow of the Japan Society for the Promotion
of Science, Chiyoda-ku, Tokyo 102-8471, Japan

Present Address:

R. Nakai
National Institute of Genetics, 1111 Yata, Mishima,
Shizuoka 411-8540, Japan

T. Abe
Graduate School of Science and Technology, Niigata University,
8050 Ikarashi 2-no-cho, Nishi-ku, Niigata 950-2181, Japan

T. Baba · H. Kagoshima · H. Niki · K. Yanagihara
Transdisciplinary Research Integration Center, 4-3-13
Toranomon, Minato-ku, Tokyo 105-0001, Japan

S. Imura · H. Kanda
National Institute of Polar Research, 10-3 Midori-cho,
Tachikawa, Tokyo 190-8518, Japan

H. Kagoshima · Y. Kohara · H. Niki
National Institute of Genetics, 1111 Yata, Mishima,
Shizuoka 411-8540, Japan

Keywords Antarctic lake · Moss pillar · Eukarya · Bacteria · RuBisCO · Phylogenetic analyses

Introduction

Antarctic freshwater lake ecosystems are generally classified as oligotrophic or ultra-oligotrophic depending on the phytoplankton biomass in lake water (Padisák 2004;

Lizotte 2008). These oligotrophic lakes are covered with ice for the majority of the year, leading to variation in the light environment depending on the ice thickness and snow cover (Vincent et al. 1998). Moreover, biologically harmful levels of solar radiation may penetrate through the ice cover and shallow water column (Vincent et al. 1998) because of low concentrations of UV-absorbing material (Morris et al. 1995). Benthic microbial communities in the lake produce carotenoids, xanthophyll, scytonemin, and mycosporine-like amino acids (Vincent et al. 1993a; Quesada et al. 1999; Hodgson et al. 2004; Tanabe et al. 2010), presumably to protect against intense light conditions (Kudoh et al. 2009). Despite this extreme environment, large pillar communities of aquatic mosses have been found in on the lake-bed near the Syowa Station located in East Antarctica (Imura et al. 1999). These pillars were distributed at depths greater than about 2 m (Imura et al. 1999). Analysis of lake environmental data obtained during a continuous year-long observation from January 2004 to February 2005 revealed that the chlorophyll *a* concentration, an indicator of primary production, was between 0.1 and 0.8 $\mu\text{g L}^{-1}$ in the lake where moss pillars were observed (Tanabe et al. 2008), thereby classifying the environment as ultra-oligotrophic.

The exterior of the moss pillars is green with adherent oxygen bubbles resulting from photosynthesis, while the interior is packed with degraded moss tissues and gives off a strong odor of sulfur compounds (Imura et al. 2000). It is thought that an oxygen gradient exists between the exterior and interior (Kudoh et al. 2003a). Thus, the pillars have distinct redox-affected sections: oxidative exteriors and reductive interiors. Nakai et al. (2012a) reported high bacterial diversity and unique genotypes, or phylotypes, in moss pillars. Some phylotypes were distributed pillar-wide, while others were section-specific. Bacterial communities differ between the exterior and interior of the pillar and are hypothesized to participate in different biogeochemical processes. Moreover, 18S rRNA genetic diversity analysis revealed that a wide range of eukaryotic phylotypes, related to fungi, ciliates, tardigrades, rotifers, and nematodes, were present in the moss pillar ecosystem formed in ultra-oligotrophic lakes (Nakai et al. 2012b). However, SSU rRNA genes do not encode a physiological function that is important in biogeochemistry, and this sequence alone does not provide information on the metabolism of the cell. Thus, the biota involved in primary production and the formation and maintenance of this ecosystem remains unknown in ultra-oligotrophic lakes.

We investigated microorganisms with the potential for CO₂ fixation in moss pillars by using genetic diversity analysis of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, E.C. 4.1.1.39)—an enzyme involved in

CO₂ fixation in the Calvin–Benson–Bassham (CBB) pathway. Since it constitutes approximately 30 % of the total protein in a plant leaf, RuBisCO is probably the most abundant protein (Chase 1993; Jensen 2000). The three known forms of RuBisCO consist of a hetero-16-unit polymer form I, a homodimer form II, and a homo-10-unit polymer form III (Badger and Bek 2008). Form I RuBisCO is generally divided into two types, green-like and red-like RuBisCOs. Green-like RuBisCO is classified into forms IA and IB, whereas red-like RuBisCO is classified into forms IC and ID based on homology of amino acid sequences, resulting in a total of four forms. Form IA RuBisCO is found primarily in proteobacteria, form IB in cyanobacteria and higher plants, form IC in proteobacteria, and form ID in heterokonts and haptophytes. Form II RuBisCO is found in proteobacteria and dinoflagellate algae. To date, form III RuBisCO has only been found in archaea (Berg et al. 2010).

The results of molecular phylogenetic analysis have been reported for a diverse range of RuBisCO genes in extreme environments, including the deep sea (Elsaied and Naganuma 2001; Elsaied et al. 2002, 2006, 2007), groundwater (Alfreider et al. 2009), caves (Chen et al. 2009), sediment from Lake Natron (Kovaleva et al. 2011), and salt lakes (Giri et al. 2004). Although the RuBisCO gene sequences from known isolated strains of Antarctic microorganisms have been reported (e.g., Liu et al. 2006), there is limited information on the genetic diversity in Antarctic moss pillars. Our data thus provide further insights into RuBisCO gene diversity among Antarctic microorganisms and the carbon cycle in Antarctic lakes.

Materials and methods

Study area

An Antarctic moss pillar specimen (diameter 22 cm; height 30 cm) was harvested on January 19, 2000, during the 42nd Japanese Antarctic Research Expedition at Lake Hotoke-Ike (69°28'S, 39°34'E), Skarvsnes, ~50 km south of Syowa Station in East Antarctica, as described in Nakai et al. (2012a). The water quality of the lake was described by Kudoh et al. (2003b).

DNA extraction

The moss pillar specimen was thawed partially and sectioned into 14 samples (seven exterior and seven interior samples) by separating the interior (blackish brown) from the exterior (green) layer and longitudinally dividing each specimen into seven horizontal sections. Exterior sections were labeled O1–O7 and interior sections were labeled

II–I7, as described previously (Nakai et al. 2012a). Sections were prepared by freeze-drying and then grinding with a sterilized mortar and pestle. Mixed microbial genomic DNA was extracted by applying a partially modified version of the bead-beating method reported by Miller et al. (1999) to 500 mg (dry weight) of each sample. Briefly, a sub-sample (100 mg dry weight) was placed in a 2-mL screw-cap microtube, with 1.2 g of sterilized 0.1-mm diameter zirconium/silica beads (BioSpec Products Inc., Bartlesville, OK, USA), 0.3 mL of phosphate buffer (100 mM NaH₂PO₄, pH 8.0), 0.3 mL of lysis buffer (10 % SDS, 100 mM NaCl, 500 mM Tris–HCl, pH 8.0), and 0.3 mL of chloroform-isoamyl alcohol (24:1). The microtube was set in a Mini Bead-Beater 8 (BioSpec Products) and shaken (3,200 rpm, 3 min). Beads and broken cell fragments in the tube were then removed by centrifugation (15,000 rpm, 5 min). The supernatant was purified using a Mag Extractor-Genome kit (Toyobo, Osaka, Japan) following the manufacturer's instructions.

PCR amplification

A PCR clone library was generated from the RuBisCO gene in the purified genomic DNA. The *cbbL* gene of form I RuBisCO was amplified with primers IAB-595f and IAB-1385r, IC-537f and IC1212r, and ID-537f and ID-1212r (Table 1). The *cbbM* gene of form II RuBisCO was amplified with primers II-537f and II-1113r. The archaeal *cbbL* gene of form III RuBisCO was amplified with primers Arch-357f and Arch-891r. We used a previously reported primer specific to the form IB RuBisCO in Bryophyta (Tsubota et al. 1999) to obtain the near-full-length RuBisCO gene sequences derived from moss, the dominant component of moss pillars. The PCR conditions are described by Tsubota et al. (1999), Elsaied and Naganuma (2001), and Elsaied et al. (2007).

Construction of clone libraries and sequence analyses

The PCR-amplified products were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) before transformation into *Escherichia coli* TOP10 (Invitrogen). The sequence of the inserted RuBisCO gene was determined using an ABI 3730XL automatic DNA sequencer (Applied Biosystems, Foster, USA). Similar sequences were grouped into operational RuBisCO units (ORUs) with >90 and 100 % identity for nucleotide and amino acid sequences, respectively, according to Elsaied and Naganuma (2001) and van der Wielen (2006). Inferred amino acid sequences for the ORUs were obtained using transeq (European Bioinformatics, <http://www.ebi.ac.uk/emboss/transeq/>). When the amino acid sequence contained the stop codon, it was excluded from further analysis. Nucleotide–nucleotide and protein–protein BLAST homology searches were conducted using the NCBI nt-database to compare the ORUs with known RuBisCOs (Altschul et al. 1997). Sequences that did not match with RuBisCO genes were excluded. We used CLUSTALX (Larkin et al. 2007) to perform alignments with ORU sequences and the closest sequences obtained from both nucleotide–nucleotide and protein–protein BLAST searches. The post-alignment sequences were used to create a phylogenetic tree by the neighbor-joining (NJ) method (Saitou and Nei 1987) using the MEGA 5.0 program (Tamura et al. 2011). The diversity of the ORUs was analyzed using the Rarefaction Calculator (<http://www.biology.ualberta.ca/jbrzusto/rarefact.php>). The ORU sequences were deposited in the DDBJ/EMBL/GenBank database under accession numbers AB695360 to AB695363 for bacterial form IA ORUs; AB695364 to AB695372 for bacterial form IB ORUs; AB695373 for a bryophytic form IB ORU; and AB695374 to AB695375 for bacterial form II ORUs.

Table 1 Primers used for PCR amplification of different RuBisCO forms

Primer	Target	Sequence	References
IAB-595f	IA and IB	5'-GAC TTC ACC AAA GAC GAC GA-3'	Elsaied and Naganuma (2001)
IAB-1385r		5'-TCG AAC TTG ATT TCT TTC CA-3'	
rbcL7	IB in Bryophytes	5'-TGG ATT TAA AGC TGG TGT TAA AG-3'	Tsubota et al. (1999)
rbcL1346hR		5'-GCA GCT AAT TCA GGA CTC C-3'	
IC-537f	IC	5'-ACS AAG CCC AAG CTG GGC CTG TCG GGC-3'	Elsaied et al. (2007)
IC1212r		5'-GAT GGT GCC GCC GAA CTG-3'	
ID-537f	ID	5'-GTA AAA CCT AAA TTA GGT YTA TCT GGT-3'	Elsaied et al. (2007)
ID-1212r		5'-AAT AGT ACC ACC ACC AAA TTG-3'	
II-537f	II	5'-ATC ATC AAR CCS AAR CTS GGC CTG CGT CCC-3'	Elsaied and Naganuma (2001)
II-1113r		5'-MGA GGT GAC SGC RCC GTG RCC RGC MCG RTG-3'	
Arch-357f	III	5'-GCH GGR AAY ATY TTY RGM ATG AAR-3'	Elsaied et al. (2007)
Arch-891r		5'-KGC NGM ATG CAT RSM NCK GTG GSC RTG-3'	

Results and discussion

Distribution of RuBisCO genes obtained from the moss pillar

The form IA/IB RuBisCO gene was amplified using the primer set IAB-595f and IAB-1385r from all 14 sections (seven exterior and seven interior) of the studied moss pillar. The near-full-length form IB RuBisCO in Bryophyta was also amplified using the primer set rbcL7 and rbcL1346hR from all 14 sections. Conversely, form II was only amplified from I2 of the lower interior layer. No form IC, ID, and III RuBisCO genes were detected by PCR in any of the sections despite several PCR runs. In addition, the archaeal 16S rRNA gene was not detected in the microfloral analysis reported by Nakai et al. (2012a) for the moss pillar ecosystem. The number of archaea with the potential for CO₂ fixation is low to non-existent, almost to the point of being undetectable by PCR.

A total of 29 PCR clone libraries were constructed for each section. We then randomly selected and sequenced 65 clones from each of the form IA/IB libraries, 9–10 clones from each of the bryophytic form IB libraries, and 45 clones from a form II library (total $N = 1,092$ clones). After determining the 1,092 clone sequences constructed from the PCR products, 884 clones were obtained for final analysis that excluded sequences containing the stop codon and sequences that did not hit with the RuBisCO gene. These 884 clones were grouped into 16 ORUs based on their ORU classification (matching >90 % of nucleotide sequences and 100 % of amino acid sequences) following to Elsaied and Naganuma (2001) and van der Wielen (2006). Four, ten, and two ORUs (out of 16) were assigned to form IA, IB, and II RuBisCO genes, respectively, by molecular phylogenetic analysis (Table 2; the distribution of each ORU in the 14 sections, results of the BLAST analysis for nucleotide identity, and amino acid identity are shown in Table S1).

There is a high probability that the 14 form I ORUs could function as CO₂ fixation enzymes because they contained unique functional motifs (DFTKDDE) (Shinozaki et al. 1983; Newman and Gutteridge 1993) specific to form I RuBisCO. The previously reported functional motifs (GGDFIKNDE) (Schneider et al. 1990) were also found in the two form II ORUs obtained in this study. The rarefaction curves for bacterial form I RuBisCO gene obtained from sections O2, O3, I3, and I5 did not reach clear saturation, indicating that further sampling of these clone libraries would have revealed little additional diversity (Fig. 1).

Diversity of form IA RuBisCO genes from proteobacteria

We obtained 342 sequences belonging to the form IA RuBisCO gene from proteobacteria (approximately 49 %

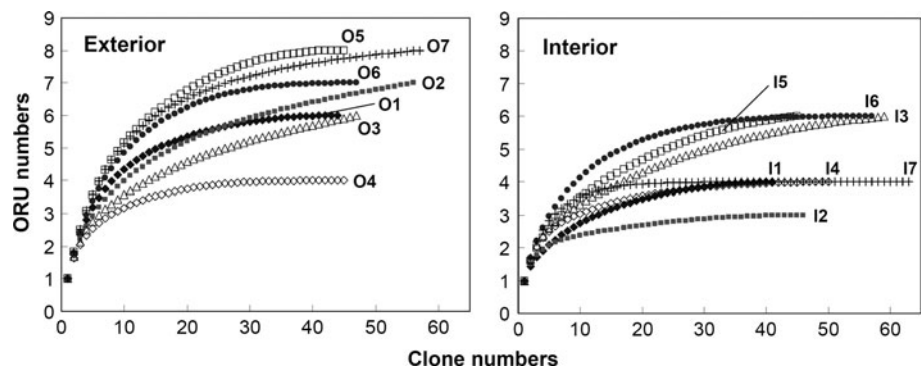
of the 702 total clones) among the clone libraries constructed with the primer set IAB-595f and IAB-1385r specific to the form IA/IB, and they were classified into four ORUs (Table 2). These were detected at roughly the same frequency as the form IB RuBisCO gene from cyanobacteria. ORU1 was most dominant (288/342 clones) of the four ORUs and was found in all 14 exterior and interior moss pillar sections (Table 2). ORU1 was most closely related to an environmental clone obtained from a tar oil-contaminated aquifer (EU926498), with 82 %_{ND} (nucleotide identity) and 93 %_{AA} (amino acid identity). The difference in identity between the nucleotide and amino acid sequence is influenced by the variability of the third base of the codon. A BLAST search for the RuBisCO genes of known isolates revealed that ORU1 was most closely related in nucleotide sequence to the RuBisCO gene of genus *Thioalkalivibrio* (CP001905) with 79 %_{ND}, and in amino acid sequence to the RuBisCO gene of genus *Bradyrhizobium* (YP_001238690) with 89 %_{AA}. ORU1 was also closely aligned on the phylogenetic tree with the genus *Bradyrhizobium* (Fig. 2). In addition, a previously reported bacterial 16S rRNA genetic diversity analysis (Nakai et al. 2012a) found phylotypes closely related to the genus *Bradyrhizobium* in all 14 exterior and interior sections. A member of the genus *Bradyrhizobium* can grow both heterotrophically, in the presence of oxygen, and anaerobically, as denitrifiers (van Berkum and Keyser 1985; Delgado et al. 2003). Moreover, some species fix carbon dioxide under microaerobic conditions using hydrogen as an electron donor (Hanus et al. 1979; Lepo et al. 1980), whereas others grow chemolithoautotrophically using thiosulfate as an electron donor (Masuda et al. 2010). The wide distribution of *Bradyrhizobium*-related sequences may be reflected by the ability to grow under aerobic, microaerobic, and anaerobic conditions.

Another surprising finding was that ORU2 (49/342 clones), found in all sections of the moss pillar, was closely related (81–85 %_{ND} and 95 %_{AA}) to the RuBisCO gene sequences from hydrothermal plumes and endosymbiotic bacteria from the deep-sea mussel *Bathymodiolus azoricus*. The phylogenetic tree indicated that it was closely aligned with a RuBisCO sequence found in endosymbiotic bacteria from deep-sea mussels (AAX48774) (Spiridonova et al. 2006) (Fig. 2). Deep-sea mussels are thought to rely on the primary production of chemoautotrophic bacteria that live symbiotically in the branchial cells of the mussels (McKiness and Cavanaugh 2005). There are three known types of symbiotic, chemoautotrophic bacteria: sulfur-oxidizing bacteria, methane-oxidizing bacteria, and both sulfur- and methane-oxidizing bacteria (McKiness and Cavanaugh 2005). A BLAST search for the RuBisCO genes of known isolates revealed that ORU2 found in this study was most closely related in nucleotide sequence

Table 2 Clone number distributions in the RuBisCO gene clone libraries and phylogenetic affiliations of ORUs

ORU	Closest amino acid sequence	Accession no.	Identity (%AA)	Coverage (%AA of hit/query)	Exterior section							Interior section							Total
					O1	O2	O3	O4	O5	O6	O7	I1	I2	I3	I4	I5	I6	I7	
RuBisCO form IA																			
ORU1	Tar oil-contaminated aquifer clone D10gl104	ACH70420	93	249/268	12	17	19	14	11	17	4	30	29	26	24	27	29	29	288
ORU2	Hydrothermal vent plume clone Suiyo (I)-8	BAD14298	95	257/270	2	3	4	3	2	4	2	7	2	4	2	3	3	8	49
ORU3	Tropical deltaic mobile mud sediment clone fg5L762	AAX21124	90	243/270													4	4	
ORU4	Tar oil-contaminated aquifer clone D10gl104	ACH70420	89	239/268							1								1
RuBisCO form IB																			
ORU5	<i>Leptolyngbya laminosa</i> ETS-08	CAV30127	97	263/270	17	25	19	24	11	5	13	2	15	24	19	10	8	17	209
ORU6	<i>Microcoleus vaginatus</i> FGP-2	ZP_08492630	98	265/270		1	1		3	4	4	2				1	3		19
ORU7	<i>Nostoc</i> sp. PCC 7120	NP_485564	97	262/270	6	4	1		2	3	13		2	5	2	10	9		57
ORU8	<i>Synechococcus</i> sp. PCC 7335	ZP_05037744	93	252/270		1			2				1		2				6
ORU9	<i>Raphidiopsis brookii</i> D9	ZP_06304977	91	247/270										2					2
ORU10	<i>Nostoc</i> sp. PCC 7120	NP_485564	83	223/270	5														5
ORU11	<i>Leptolyngbya lamnosa</i> ETS-08	CAV30127	96	259/270	2	5	3	4	11	12	14								51
ORU12	<i>Thermosynechococcus elongatus</i> BP-1	NP_682296	93	252/270					3	2									5
ORU13	<i>Leptolyngbya lamnosa</i> ETS-08	CAV30127	93	250/270							6								6
RuBisCO form IB in bryophytes																			
ORU14	<i>Leptobryum</i> sp. L_Hotoke	BAL43045	100	446/446	10	10	9	10	10	10	9	10	10	10	9	10	10	10	137
RuBisCO form II																			
ORU15	Tar oil-contaminated aquifer clone D10M33	ACH70392	93	124/133									35						35
ORU16	Polluted groundwater clone L4C16	ABY77470	97	129/133									10						10

Fig. 1 Rarefaction curves for the expected number of ORUs for bacterial form I RuBisCO gene based on grouping of clones that have >90 and 100 % identity for nucleotide and amino acid sequences, respectively



to the sulfur-oxidizing bacteria genus *Thiohalophilus* RuBisCO gene (GQ888587) with 82 %_{ND}, and in amino acid sequence to the sulfur-oxidizing bacteria genus

Acidithiobacillus RuBisCO gene (P0C917) with 89 %_{AA}. Hydrogen sulfide gas was undetected in the water column and at the surface of the phytobenthos in Lake Hotoke-Ike,

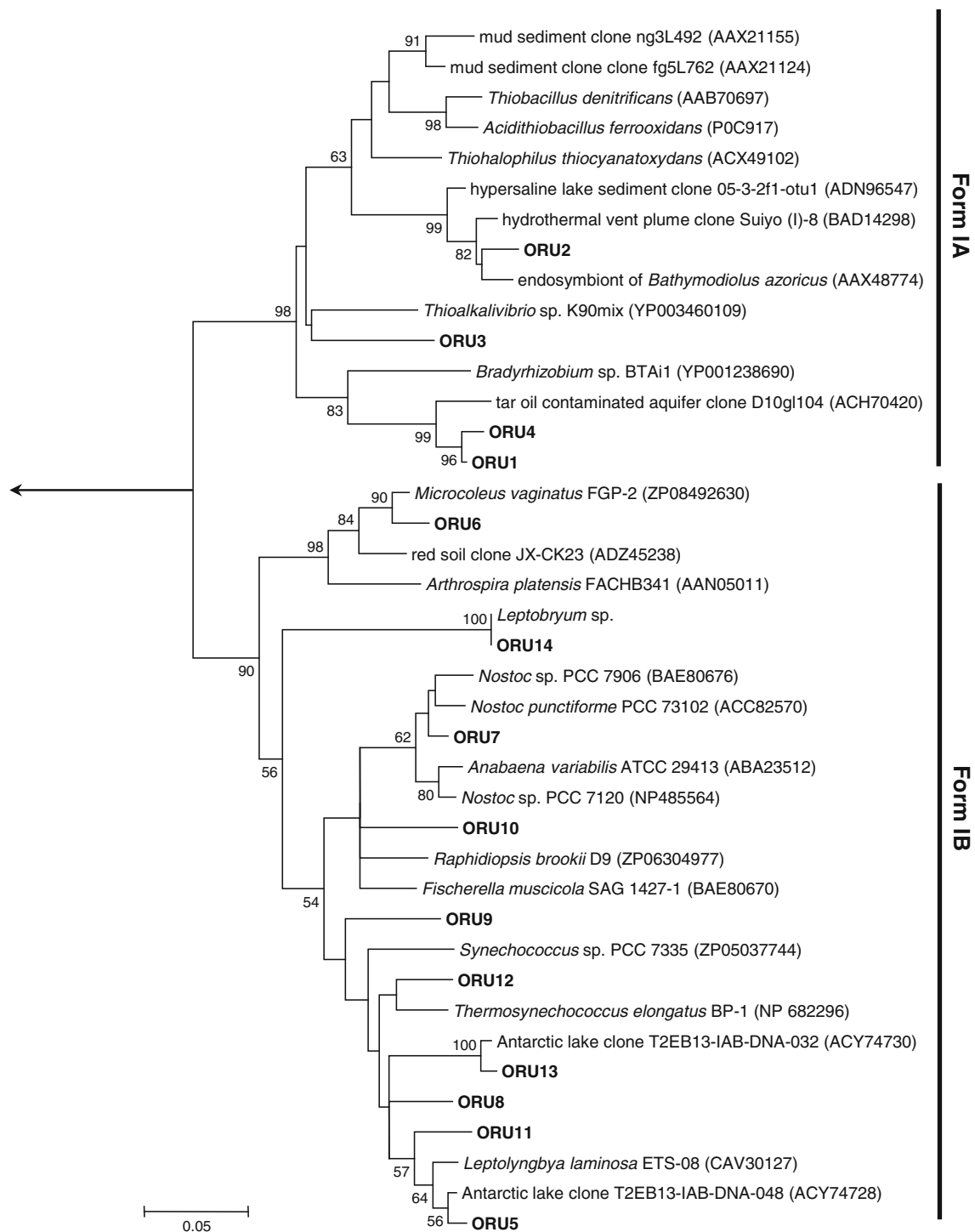


Fig. 2 Phylogenetic tree of ORUs of the forms IA and IB RuBisCO amino acid sequences obtained from aquatic moss pillars. Evolutionary history was inferred by using the neighbor-joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches

corresponding to partitions reproduced in <50 % bootstrap replicates were collapsed. The RuBisCO amino acid sequence of *Olisthodiscus luteus* (BAF80663) was used as outgroup. Accession numbers are shown in *parentheses*

but $\geq 1 \mu\text{g g}^{-1}$ (wet weight) of hydrogen sulfide gas was detected from deeper than 5 mm within the phytobenthos mat samples (Kudoh et al. 2009). The low concentration at

the surface is assumed due to the rapid oxidation of hydrogen sulfide by sulfur-oxidizing bacteria. Thus, sulfur-oxidizing bacteria may contribute to primary production by

synthesizing organic matter via the oxidation of hydrogen sulfide in the pillar.

The two remaining ORUs shared 83 %_{ND} and 90 %_{AA}, and 81 %_{ND} and 89 %_{AA} identity, respectively, with an environmental clone obtained from tropical muddy sediments (AY773062) and an environmental clone obtained from an aquifer (EU926498).

Our results suggest that proteobacteria are distributed throughout the moss pillar, where they may play a role in primary production via chemosynthesis in the moss pillar ecosystem by fixing carbon dioxide without relying on light energy. In addition, RuBisCO genes with extremely high identity were found at the deep-sea environments and in Antarctic lakes, two environments with widely differing conditions. Bacteria with this type of RuBisCO gene may be present in other Antarctic lakes since they are thought to be widely distributed.

Diversity in form IB RuBisCO genes from mosses and cyanobacteria

We obtained 137 sequences from the clone libraries that were constructed with the primer set rbcL7 and rbcL1346hR specific to the form IB RuBisCO gene in Bryophyta, and they were classified into a single ORU (Table 2). ORU14, considered to be the major component species of the pillar, shared 100 % nucleotide sequence identity with the RuBisCO gene sequence of *Leptobryum* sp. isolated from Lake Hotoke-Ike (AB690288) where the moss pillars were found. This agrees with the report by Imura et al. (1999) that *Leptobryum* sp. comprises the main structural component of moss pillars. However, the sequences related to *Bryum*, a minor structural species in moss pillars, were not obtained. Previous reports have shown that *Leptobryum* species are the primary mosses in moss pillars, whereas *Bryum pseudotriquetrum* is a minor or absent (Ohtani et al. 2001). Moreover, 18S rRNA gene-based diversity analysis (Nakai et al. 2012b) revealed clones closely related to *Leptobryum* 18S rRNA genes in all moss pillar sections, whereas *Bryum*-related clones were not found. Thus, *Bryum* sp. may have been absent in the moss pillar samples used in this study.

We obtained 360 sequences belonging to the form IB RuBisCO gene from cyanobacteria (approximately 51 % of total 702 clones) among the clone libraries constructed with the primer set IAB-595f and IAB-1385r specific to the form IA/IB, and they were classified into nine ORUs (Table 2). Excluding ORU10, these ORUs shared comparatively high identity in the range of 81–99 %_{ND} and 91–98 %_{AA} with the RuBisCO gene sequences of cyanobacteria such as *Anabaena*, *Arthrospira*, *Fischerella*, *Leptolyngbya*, *Microcoleus*, *Nostoc*, *Raphidiopsis*, *Synechococcus*, and *Thermosynechococcus*. Cyanobacteria

form mats at the bottom of Antarctic lakes and they are thought to be the dominant primary producers on the lake-bottom (Vincent 1988, 2000; Quesada et al. 2008). ORU5 was the most dominant (209/360 clones) of these nine ORUs and was found in all 14 exterior and interior sections of the moss pillar (Table 2). ORU5 was highly homologous with the RuBisCO gene sequence of known species *Leptolyngbya laminosa* ETS-08, with 90 %_{ND} and 97 %_{AA}. ORU5 was closely aligned on the phylogenetic tree with an environmental clone obtained from an Antarctic lake, Lake Bonney, in the McMurdo Dry Valleys (ACY74728) (Fig. 2). A number of species of cyanobacteria, including *Lyngbya*, *Oscillatoria*, *Nostoc*, and *Phormidium* spp., were detected by using microscopy in samples of the lake-bed from Lake Bonney, the surface of which is covered with ice year-round (Vincent et al. 1993b; Wing and Prisco 1993; Paerl and Pinckney 1996). Our data demonstrate that RuBisCO genes from geographically distant Antarctic lakes, with Lake Bonney and Hotoke-Ike located approximately 3,000 km apart, are phylogenetically related. ORU13 was close on the phylogenetic tree to an environmental clone found in Lake Bonney (ACY74730), and it formed one independent cluster.

With 87 %_{ND} and 96 %_{AA} identity with the RuBisCO gene sequence for the genus *Leptolyngbya*, ORU11 was found exclusively in the exterior layer and it clustered with the widely distributed ORU5 on the phylogenetic tree. Thus, the distribution characteristics differed even among phylogenetically related ORUs. The phylotype related to the genus *Leptolyngbya* were also found exclusively in the exterior layer of moss pillars in a previous bacterial 16S rRNA genetic diversity analysis (Nakai et al. 2012a), indicating a high probability that some *Leptolyngbya* bacterial species are specific to the exterior layer of the pillar. ORU10 differed from the other eight ORUs as they shared homology with all RuBisCO gene sequences in the database. It was most closely related to the *Nostoc* RuBisCO gene with 84 %_{ND} and 83 %_{AA}. It was found only in the deepest part of the exterior layer (O1) of the moss pillar and formed an independent cluster on the phylogenetic tree (Fig. 2).

Diversity and phylogeny of form II RuBisCO genes from proteobacteria

We obtained 45 sequences from the clone libraries constructed with the primer set II-537f and II-1113r specific to the form II RuBisCO gene, and they were classified into two ORUs (Table 2). Form II was only detected from I2 of the lower interior layer. The first, ORU15 (35/45 clones), was most closely related in nucleotide sequence to an environmental clone from Movile Cave in Romania (FJ604806) with 92 %_{ND}, and in amino acid sequence to an

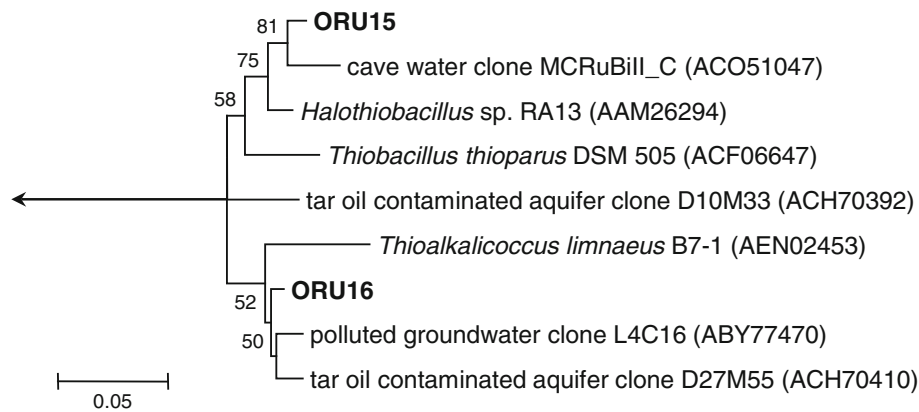


Fig. 3 Phylogenetic tree of ORUs of the form II RuBisCO amino acid sequences obtained from aquatic moss pillars. Evolutionary history was inferred by using the neighbor-joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches

corresponding to partitions reproduced in <50 % bootstrap replicates were collapsed. The RuBisCO amino acid sequence of *Nostoc punctiforme* PCC73102 (ACC82570) was used as outgroup. Accession numbers are shown in *parentheses*

environmental clone from a tar oil-contaminated aquifer (ACH70392) with 93 %_{AA}. A BLAST search of known RuBisCO gene isolates revealed that this ORU was most closely related to the RuBisCO gene sequence of the obligately chemolithoautotrophic, sulfur-oxidizing bacterium *Halothiobacillus* sp. RA13 (AY099399), with 90 %_{ND} and 95 %_{AA}. Salt is not essential for the growth of *Halothiobacillus* sp., although it is known to tolerate a high salt concentration (4 M NaCl and 0.25 M sodium thiosulfate) (Kelly and Wood 2000). The freshwater lakes inhabited by *Leptobryum* sp., the dominant component of moss pillars, have electronic conductivity in the range of 15.0–390.0 ms/mm (Imura et al. 2003). This highest value is close to one-tenth of the value in seawater.

The second, ORU16 (10/45 clones), was related to the RuBisCO gene sequence of the Chromatiales *Thioalkalicoccus limnaeus* B7-1 (AEN02453), with 86 %_{ND} and 95 %_{AA}. This result was supported by the phylogenetic tree (Fig. 3). *T. limnaeus* is an alkaliphilic bacterium isolated from Lake Natron in southeast Siberia, Russia with a pH range of 9.5–10.1 (Bryantseva et al. 2000), which differs significantly from the 7–8-pH range of Lake Hotoke-Ike (Kimura et al. 2010). Purple sulfur bacteria, of the order Chromatiales, generally engage in photoautotrophic metabolism using reduced inorganic sulfur compounds (e.g., hydrogen sulfide) as electron donors. *Thioalkalicoccus* sp. contains bacteriochlorophyll *b* that functions intracellularly as a photosynthetic pigment (Bryantseva et al. 2000). Thus, this group can only grow in limited areas in anaerobic environments that receive light and contain a stable supply of hydrogen sulfide. The detection of the form II ORU related to Chromatiales may indicate that light is available to phototrophs even in the lower interior part of the pillar.

Conclusion

We studied the distribution and diversity of all forms (forms IA, IB, IC, ID, II, and III) of the RuBisCO large subunit-encoding gene in a whole moss pillar. Proteobacterial form IA ORUs were present at the same frequency as cyanobacterial form IB ORUs. Surprisingly, the form IA ORU, which is closely related to the sequences from deep-sea environments such as hydrothermal plumes and endosymbiotic bacteria from deep-sea mussels, was detected in all moss pillar sections. The form IB ORU belonging to Bryophyta, considered to be derived from moss, was 100 % identical to the RuBisCO gene sequence from *Leptobryum* sp. In addition, some cyanobacterial ORUs were found exclusively in the exterior layer of the pillar, whereas form II ORUs related to obligately chemolithoautotrophic sulfur oxidizers, and purple sulfur bacteria were detected exclusively in the interior layer. This layer-specific distribution of ORUs reflects the double-layered structure of an oxidative exterior and a reductive interior layer in the pillars. This is the first report demonstrating that bacteria with the potential for CO₂ fixation and chemoautotrophy are present in the Antarctic moss pillar ecosystem. CO₂ fixation by chemoautotrophic bacteria is likely to be especially important during the polar night months in Antarctica. This could be verified by investigating the types and amounts of RuBisCO genes expressed in the field using a RNA-based PCR clone library analysis, or metatranscriptome and metaproteome analysis.

Acknowledgments We gratefully acknowledge the members of the 42nd Japanese Antarctic Research Expedition (JARE). We thank Kazuko Ohishi and Tadasu Shin-i for their technical assistance. This study was supported by the National Polar Research Institute and the Transdisciplinary Research Integration Center, Research Organization

of Information and Systems, Japan. This study also supported by JSPS Research Fellowships for Young Scientists (No. 11J30005). The study was conducted as part of the Microbiological and Ecological Responses to Global Environmental Changes in Polar Regions (MERGE) program during the International Polar Year of 2007–2008.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

- Alfreider A, Vogt C, Geiger-Kaiser M, Psenner R (2009) Distribution and diversity of autotrophic bacteria in groundwater systems based on the analysis of RuBisCO genotypes. *Syst Appl Microbiol* 32:140–150
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Badger MR, Bek EJ (2008) Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO₂ acquisition by the CBB cycle. *J Exp Bot* 59:1525–1541
- Berg IA, Kockelkorn D, Ramos-Vera WH, Say RF, Zarzycki J, Hügler M, Alber BE, Fuchs G (2010) Autotrophic carbon fixation in archaea. *Nat Rev Microbiol* 8:447–460
- Bryantseva IA, Gorlenko VM, Kompantseva EI, Imhoff JF (2000) *Thioalkalicoccus limnaeus* gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium with bacteriochlorophyll *b*. *Int J Syst Evol Microbiol* 50:2157–2163
- Chase MW (1993) Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbcl*. *Ann Missouri Bot Gard* 80:528–580
- Chen Y, Wu LQ, Boden R, Hillebrand A, Kumaresan D et al (2009) Life without light: microbial diversity and evidence of sulfur- and ammonium-based chemolithotrophy in Movile Cave. *ISME J* 3:1093–1104
- Delgado MJ, Bonnard N, Tresierra-Ayala A, Bedmar EJ, Muller P (2003) The *Bradyrhizobium japonicum* *napEDABC* genes encoding the periplasmic nitrate reductase are essential for nitrate respiration. *Microbiology* 149:3395–3403
- Elsaied H, Naganuma T (2001) Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* 67:1751–1765
- Elsaied H, Kimura H, Naganuma T (2002) Molecular characterization and endosymbiotic localization of the gene encoding ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) form II in the deep-sea vestimentiferan trophosome. *Microbiology* 148:1947–1957
- Elsaied HE, Kaneko R, Naganuma T (2006) Molecular characterization of a deep-sea methanotrophic mussel symbiont that carries a RuBisCO gene. *Mar Biotech* 8:511–520
- Elsaied HE, Kimura H, Naganuma T (2007) Composition of archaeal, bacterial, and eukaryal RuBisCO genotypes in three Western Pacific arc hydrothermal vent systems. *Extremophiles* 11:191–202
- Giri BJ, Bano N, Hollibaugh JT (2004) Distribution of RuBisCO genotypes along a redox gradient in Mono Lake, California. *Appl Environ Microbiol* 70:3443–3448
- Hanus FJ, Maier RJ, Evans HJ (1979) Autotrophic growth of H₂-uptake-positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. *Proc Natl Acad Sci USA* 76:1788–1792
- Hodgson DA, Vyverman W, Verleyen E, Sabbe K, Leavitt PR et al (2004) Environmental factors influencing the pigment composition of in situ benthic microbial communities in east Antarctic lakes. *Aquat Microb Ecol* 37:247–263
- Imura S, Bando T, Saito S, Seto K, Kanda H (1999) Benthic moss pillars in Antarctic lakes. *Polar Biol* 22:137–140
- Imura S, Takahashi H, Nakamura T (2000) Benthic moss pillars (Koke Bouzu) in Antarctic lakes—Analysis of colonization and growth by ¹⁴C dating. *Sum Res using AMS, Nagoya Univ XI*:176–183 (in Japanese with English Abstract)
- Imura S, Bando S, Seto K, Ohtani S, Kudoh S, Kanda H (2003) Distribution of aquatic mosses in the Syowa Coast region, East Antarctica. *Polar Biosci* 16:1–10
- Jensen RG (2000) Activation of Rubisco regulates photosynthesis at high temperature and CO₂. *Proc Natl Acad Sci USA* 97:12937–12938
- Kelly DP, Wood AP (2000) Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *Int J Syst Evol Microbiol* 50:511–516
- Kimura S, Ban S, Imura S, Kudoh S, Matsuzaki M (2010) Limnological characteristics of vertical structure in the lakes of Syowa oasis, East Antarctica. *Polar Sci* 3:262–271
- Kovaleva OL, Tourova TP, Muzyer G, Kolganova TV, Sorokin DY (2011) Diversity of RuBisCO and ATP citrate lyase genes in soda lake sediments. *FEMS Microbiol Ecol* 75:37–47
- Kudoh S, Tuchiya Y, Ayukawa E, Imura S, Kanda H (2003a) Ecological studies of aquatic moss pillars in Antarctic lakes. 1: macro structure and carbon, nitrogen and chlorophyll *a* contents. *Polar Biosci* 16:11–22
- Kudoh S, Watanabe K, Imura S (2003b) Ecological studies of aquatic moss pillars in Antarctic lakes. 2: temperature and light environment at the moss habitat. *Polar Biosci* 16:23–32
- Kudoh S, Tanabe Y, Matsuzaki M, Imura S (2009) In situ photochemical activity of the phytobenthic communities in two Antarctic lakes. *Polar Biol* 32:1617–1627
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA et al (2007) Clustal W and clustal X version 2.0. *Bioinformatics* 23:2947–2948
- Lepo JE, Hanus FJ, Evans HJ (1980) Chemoautotrophic growth of hydrogen-uptake-positive strains of *Rhizobium japonicum*. *J Bacteriol* 141:664–670
- Liu CL, Huang XH, Wang XL, Zhang XC, Li GY (2006) Phylogenetic studies on two strains of Antarctic ice algae based on morphological and molecular characteristics. *Phycologia* 45:190–198
- Lizotte MP (2008) Phytoplankton and primary production. In: Vincent WF, Laybourn-Parry J (eds) *Polar lakes and rivers*. Oxford University Press, Oxford, pp 157–178
- Masuda S, Eda S, Ikeda S, Mitsui H, Minamisawa K (2010) Thiosulfate-dependent chemolithoautotrophic growth of *Bradyrhizobium japonicum*. *Appl Environ Microbiol* 76:2402–2409
- McKiness ZP, Cavanaugh CM (2005) The ubiquitous mussel: *Bathymodiolus* aff. *brevior* symbiosis at the Central Indian Ridge hydrothermal vents. *Mar Ecol Prog Ser* 295:183–190
- Miller DN, Bryant JE, Madsen EL, Ghiorse WC (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl Environ Microbiol* 65:4715–4724
- Morris DP, Zagarese H, Williamson CE, Balseiro EG, Hargreaves BR et al (1995) The attenuation of solar UV radiation in lakes and

- the role of dissolved organic carbon. *Limnol Oceanogr* 40:1381–1391
- Nakai R, Abe T, Baba T, Imura S, Kagoshima H, Kanda H, Kanekiyo A, Kohara Y, Koi A, Nakamura K, Narita T, Niki H, Yanagihara K, Naganuma T (2012a) Microflora of aquatic moss pillars in a freshwater lake, East Antarctica, based on fatty acid and 16S rRNA gene analyses. *Polar Biol* 35:425–433
- Nakai R, Abe T, Baba T, Imura S, Kagoshima H, Kanda H, Kohara Y, Koi A, Niki H, Yanagihara K, Naganuma T (2012b) Eukaryotic phylotypes in aquatic moss pillars inhabiting a freshwater lake in East Antarctica, based on 18S rRNA gene analysis. *Polar Biol* (published online before print). doi:10.1007/s00300-012-1188-1
- Newman J, Gutteridge S (1993) The X-ray structure of *Synechococcus* ribulose-bisphosphate carboxylase/oxygenase-activated quaternary complex at 2.2 Å resolution. *J Biol Chem* 268:25876–25886
- Ohtani S, Kudoh S, Tsuchiya Y, Suyama K, Imura S (2001) Species composition of freshwater algae in benthic moss pillars in lakes, Skarvsnes, Antarctica. Abstracts for XXIV Symp Polar Biol Tokyo, Natl Inst Polar Res, p 77
- Padisák J (2004) Phytoplankton. In: Sullivan PE, Reynolds CS (eds) *The Lakes handbook. Limnology and limnetic ecology*, vol 1. Blackwell, Oxford, pp 251–308
- Paerl HW, Pinckney JL (1996) Ice aggregates as a microbial habitat in Lake Bonney, Dry Valley Lakes, Antarctica: nutrient-rich microzones in an oligotrophic ecosystem. *Antarctic J USA* 31:220–222
- Quesada A, Vincent WF, Lean DRS (1999) Community and pigment structure of Arctic cyanobacterial assemblages: the occurrence and distribution of UV-absorbing compounds. *FEMS Microbiol Ecol* 28:315–323
- Quesada A, Fernandez-Valiente E, Hawes I, Howard-Williams C (2008) Benthic primary production in polar lakes and rivers. In: Vincent WF, Laybourn-Parry J (eds) *Polar lakes and rivers*. Oxford University Press, Oxford, pp 179–196
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Schneider G, Lindqvist Y, Lundqvist T (1990) Crystallographic refinement and structure of ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum Rubrum* at 1.7 Å resolution. *J Mol Biol* 211:989–1008
- Shinozaki K, Yamada C, Takahata N, Sugiura M (1983) Molecular cloning and sequence analysis of the cyanobacterial gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Proc Natl Acad Sci USA* 80:4050–4054
- Spiridonova EM, Kuznetsov BB, Pimenov NV, Turova TP (2006) Phylogenetic characterization of endosymbionts of the hydrothermal vent mussel *Bathymodiolus azoricus* by analysis of the 16S rRNA, *cbbL*, and *pmoA* genes. *Mikrobiologiya* 75:798–806
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Tanabe Y, Kudoh S, Imura S, Fukuchi M (2008) Phytoplankton blooms under dim and cold conditions in freshwater lakes of East Antarctica. *Polar Biol* 31:199–208
- Tanabe Y, Ohtani S, Kasamatsu N, Fukuchi M, Kudoh S (2010) Photophysiological responses of phytobenthic communities to the strong light and UV in Antarctic shallow lakes. *Polar Biol* 33:85–100
- Tsubota H, Nakao N, Arikawa T, Yamaguchi T, Higuchi M, Deguchi H, Seki T (1999) A preliminary phylogeny of Hypnales (Musci) as inferred from chloroplast *rbcL* sequence data. *Bryol Res* 7:233–248
- van Berkum P, Keyser HH (1985) Anaerobic growth and denitrification among different serogroups of soybean rhizobia. *Appl Environ Microbiol* 49:772–777
- van der Wielen PWJJ (2006) Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes in the MgCl₂-dominated deep hypersaline anoxic basin discovery. *FEMS Microbiol Lett* 259:326–331
- Vincent WF (1988) *Microbial ecosystems of Antarctica*. Cambridge University Press, Cambridge
- Vincent WF (2000) Cyanobacterial dominance in the polar regions. In: Whitton BA, Potts M (eds) *The ecology of cyanobacteria: their diversity in time and space*. Kluwer, Dordrecht, pp 321–340
- Vincent WF, Downes MT, Castenholz RW, Howardwilliams C (1993a) Community structure and pigment organization of cyanobacteria-dominated microbial mats in Antarctica. *Eur J Phycol* 28:213–221
- Vincent WF, Castenholz RW, Downes MT, Howardwilliams C (1993b) Antarctic cyanobacteria: light, nutrients and photosynthesis in the microbial mat environment. *J Phycol* 29:745–755
- Vincent WF, Rae R, Laurion I, Howard-Williams C, Priscu JC (1998) Transparency of Antarctic ice-covered lakes to solar UV radiation. *Limnol Oceanogr* 43:618–624
- Wing KT, Priscu JC (1993) Microbial communities in the permanent ice cap of Lake Bonney, Antarctica: relationships among chlorophyll-*a*, gravel and nutrients. *Antarctic J USA* 28:246–249